

**OSTEOACTIVIN PROTEIN AND NUCLEIC ACIDS ENCODING THE SAME,
COMPOSITIONS AND METHODS OF STIMULATING BONE
DIFFERENTIATION**

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FIELD OF THE INVENTION

The present invention relates to the identification of an isolated, full-length rat nucleic acid molecule encoding an osteoactivin protein, therapeutic compositions comprising an osteoactivin protein, and methods for using the nucleic acid molecules and proteins for stimulating bone differentiation. The invention also relates to methods for treating bone disorders, including osteopetrosis and osteoporosis.

BACKGROUND OF THE INVENTION

The formation and maintenance of the vertebrate skeleton requires the interactions of many cell types and growth factors and other molecules. The past decade has witnessed an explosive growth in the general understanding of growth factors and other proteins that mediate the complex coordination of bone formation and bone resorption by these different cell types in skeletal modeling and remodeling (Popoff and Marks, *Oral and Maxillofacial Clinics of North America* 9:563-579 (1997)).

In general, the bone remodeling cycle involves a complex series of sequential steps that are highly regulated. The initial "activation" phase of bone remodeling begins early in fetal life and is dependent on the effects of local and systemic growth factors on mesenchymal cells of the osteoblast lineage (Eriksen, *Endocrinol. Rev.* 7:379-408 (1986)). These cells interact with hematopoietic precursors to form osteoclasts in the "resorption" phase. This leads to the differentiation, migration and fusion of the large multinucleated osteoclasts. These cells attach to the mineralized bone surface and initiate resorption by the secretion of hydrogen ions and lysosomal enzymes. Osteoclastic resorption produces irregular scalloped cavities on bone surface. Once the osteoclasts have completed their work of bone removal, there is a "reversal" phase during which

mononuclear cells, which may be of the macrophage lineage, are present on the bone surface. These cells further degrade collagen, deposit proteoglycans, and release growth factors that signal the initiation of the “formation” phase. During the final formation phase of the remodeling cycle, the cavity created by resorption can be completely filled in with successive layers of osteoblasts, which differentiate from their mesenchymal precursors and lay down a mineralizable matrix. (Raisz, *Clin. Chem.* 45:1353-1358 (1999)).

With bone disorders associated with decreased bone mass, osteoclastic resorption outweighs osteoblastic bone formation, resulting in bone loss. While treatments that stimulate bone formation would be beneficial in treating or preventing bone loss, current therapies are suboptimal (Canalis, *J. Clin. Invest.* 106:177-179 (2000); Raisz, *J. Bone Min. Metab* 17:79-89 (1999)).

An animal model useful in bone studies is the *osteopetrosis (op)* mutation in the rat. Osteopetrosis describes a group of congenital bone disorders that are characterized by a generalized increase in skeletal mass resulting from a primary defect in osteoclast-mediated bone resorption (Popoff and Schneider, *Molec. Med. Today* 2:349-358 (1996)). Numerous osteopetrotic mutations have been described in other species, including human and mouse. The bone that is formed as the skeleton develops and grows in animals with this mutation is not resorbed, resulting in the failure to develop bone marrow cavities. The osteopetrotic mutations are pathogenetically heterogeneous since the point at which osteoclast development or activation is intercepted differs for each mutation (Popoff and Marks, *Bone* 17:437-445 (1995)). Although osteoclast hypofunction is universal among the osteopetrotic mutations, genetic abnormalities involving osteoblast development/function (*i.e.*, bone formation), mineral homeostasis and the immune and endocrine systems have also been reported within this disorder (Seifert *et al.*, *Clin. Orthop.* 294:23-33 (1993)).

To date, pharmaceutical approaches to managing osteoporosis or osteopetrosis are of limited effectiveness. Therefore, alternative therapies are needed to modulate bone cell differentiation and bone formation, and to treat bone disorders such as osteoporosis and osteopetrosis.

SUMMARY OF THE INVENTION

The present invention is based, in part, on the discovery of a novel rat gene encoding an osteoactivin protein. The nucleotide sequence of full-length cDNA of the gene is shown in SEQ ID NO:1. The nucleotide sequence of the cDNA encoding the osteoactivin protein is shown in nucleotides 115 to 1,830 of SEQ ID NO:1 and the corresponding amino acid sequence of the osteoactivin protein is shown in SEQ ID NO:2. The polynucleotide sequence of the cDNA encoding the osteoactivin protein lacking the signal sequence is shown in nucleotides 181-1830 of SEQ ID NO:1 and the corresponding osteoactivin polypeptide lacking the signal sequence is from amino acid residues 23-572 of SEQ ID NO:2. The claimed invention also relates to antibodies which recognize one or more epitopes of the osteoactivin protein. The claimed invention provides therapeutic compositions comprising (i) a nucleic acid molecule encoding an osteoactivin protein, (ii) an osteoactivin protein, or (iii) an antibody to an osteoactivin protein. These therapeutic compositions are useful to treat bone disorders and to stimulate bone formation and bone cell differentiation.

Accordingly, in one aspect, the invention is directed to molecules encoding an osteoactivin protein. One embodiment of this aspect is a nucleic acid molecule encoding a rat osteoactivin protein having a molecular weight of 63.8 kilodaltons (kD), wherein said osteoactivin protein stimulates bone cell differentiation. In a related embodiment of this aspect, the invention encompasses a full-length nucleic acid molecule which encodes an osteoactivin protein, wherein said nucleic acid comprises the nucleic acid sequence of SEQ ID NO:1. In another embodiment, the invention provides a nucleic acid molecule encoding an osteoactivin protein, wherein said nucleic acid hybridizes to the complement of SEQ ID NO:1 under moderately stringent conditions. In a preferred embodiment, the nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence of SEQ ID NO:1 is described. In some embodiments, the nucleic acid molecule encodes a polypeptide comprising SEQ ID NO:2. The invention also embodies the nucleic acid molecule encoding an osteoactivin polypeptide comprising amino acid residues 23-572 of SEQ ID NO:2. In other embodiments, the invention provides a nucleic acid encoding an

osteoactivin protein, wherein said nucleic acid comprises from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1. Other embodiments of the invention provide a polynucleotide encoding an osteoactivin protein lacking the leader sequence, wherein said polynucleotide comprises from nucleic acid residues from 181 to 1830 of SEQ ID NO:1. In still other embodiments, the invention provides a nucleic acid encoding an osteoactivin protein, wherein said nucleic acid molecule hybridizes to the complement of nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1 under moderately stringent conditions. In yet another embodiment of this aspect, the invention further provides a nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1.

As used herein, the term "nucleic acid molecule" includes DNA molecules (*e.g.*, a cDNA or genomic DNA) and RNA molecules (*e.g.*, an mRNA) and analogs of the DNA or RNA generated, *e.g.*, by the use of nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. Nucleic acid molecules include naturally occurring nucleic acid molecules which are separated from other molecules which are present in the natural source of the nucleic acid. For example, a nucleic acid molecule includes genomic DNA which is separated from the chromosome with which the genomic DNA is naturally associated. Preferably, a naturally occurring nucleic acid molecule is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and/or 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than 5 kilobases (kb), 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of 5' and/or 3' nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an isolated nucleic acid molecule, such as a cDNA molecule, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "osteoactivin protein" refers to a protein including the amino acid sequence of SEQ ID NO:2, the murine osteoactivin protein

homolog, nmb, of SEQ ID NO:5, the human osteoactivin protein homolog, nmb, of SEQ ID NO:6, and the amino acid sequence comprising amino acid residues 23-572 of SEQ ID NO:2. Further, an osteoactivin protein has at least 50% sequence identity, preferably 70% sequence identity, and more preferably 90% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, or SEQ ID NO:6, and stimulates bone cell differentiation or bone formation. Preferably, the osteoactivin protein is naturally occurring in a mammalian species.

As used herein, "stimulates bone cell differentiation" means any increase in bone cell number or size, including without limitation, the increase in the rate of bone cell division or precursor bone cell recruitment from the stem cells or bone marrow cells, and an increase in bone cell size. Such bone cell differentiation can be measured by well known cell proliferation assays (*e.g.*, ^3H -thymidine incorporation) and bone differentiation assays (*e.g.*, Owen, et al., *J. Cell Physiol.* 143:420-30 (1990)).

As used herein, by "stimulates bone formation" is meant the recruitment of osteoblasts or osteoblast precursors to a bone site, which results in differentiation of the cells into mature osteoblasts and their secretion of collagenous matrix which mineralizes into bone matter and increases bone mass at the site. This term also encompasses the increased production and secretion of collagenous matrix by mature osteoblasts.

As used herein, the term "hybridizes under moderately stringent conditions" describes conditions for hybridization and washing. Stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. An example of moderately stringent hybridization conditions is hybridization in 50% formamide 6X SSC at 42°C, followed by one or more washes in 0.2X SSC, 0.1% sodium dodecyl sulfate (SDS) at 55°C. In some preferable embodiments, an isolated nucleic acid molecule of the invention that hybridizes under moderately stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule.

By two nucleic acid molecules being "complementary" to one another or hybridizing to a "complement" of another nucleic acid molecule is meant that the

first nucleic acid molecule (*e.g.*, an oligonucleotide) is able to form Watson-Crick base pair hydrogen bonds (*i.e.*, hybridize) with the second nucleic acid molecule to form a duplex.

As used herein, a percent "sequence identity" refers to a calculation of "homology" or "identity" between two different nucleic acid or amino acid sequences (the terms are used interchangeably herein) when the sequences are aligned and compared. The percent sequence identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

In another aspect of the invention, the invention features an isolated and substantially pure osteoactivin protein, or polypeptide fragment thereof. One preferred embodiment of this aspect of the invention is an isolated and substantially pure rat osteoactivin protein, or polypeptide fragment thereof, wherein said protein comprises the amino acid sequence of SEQ ID NO:2. In another embodiment, the invention provides an isolated and substantially pure, non-human, non-murine osteoactivin protein, or polypeptide fragment thereof, having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2, wherein said osteoactivin protein or polypeptide fragment thereof stimulates bone cell differentiation or bone formation.

An "isolated" or "purified" osteoactivin protein or polypeptide is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. In one embodiment, the language "substantially free" means preparation of osteoactivin protein having less than 30%, 20%, 10% and more preferably less than 5% (by weight), of non-osteoactivin protein (also referred to herein as a "contaminating protein"), or of chemical precursors or non-osteoactivin compounds. When the osteoactivin protein, or biologically active portion thereof, is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, the culture medium represents less than 20%, more preferably less than 10%, and most preferably less than 5% of the volume of the protein preparation. The invention

includes isolated or purified preparations of at least 0.01 milligrams, at least 0.1 milligrams, at least 1.0 milligrams, and at least 10 milligrams by weight.

Also included, in another aspect of the invention, are expression vectors containing nucleic acid molecules encoding an osteoactivin protein or polypeptide fragment therein. In one embodiment, the invention features a biologically functional expression vector comprising a nucleic acid sequence encoding an osteoactivin protein, or biologically active polypeptide fragment thereof, wherein said osteoactivin protein comprises the amino acid sequence of SEQ ID NO:2, or has at least 90% sequence identity to the amino acid sequence of SEQ ID NO:2, and which stimulates bone cell differentiation or bone formation.

with the nucleic acid sequence from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1. In each of these embodiments, the vector may be a plasmid or a viral vector.

As used herein, the term "vector" refers to a composition capable of carrying a nucleic acid molecule to its target. Vectors include liposomes and nucleic acid molecules capable of transporting another nucleic acid to which it has been linked. Such nucleic acid vectors include plasmids, cosmids, or viral vectors. The nucleic acid vector can be capable of autonomous replication or it can integrate into a host DNA. Viral vectors include, *e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses. A "biologically functional expression vector" as used herein refers to a vector used to incorporate nucleic acid molecules of the invention, including an osteoactivin-encoding nucleic acid, in a form suitable for expression in a host cell.

In yet another aspect, the invention features immunoglobulins such as antibodies and antigen-binding fragments thereof, that recognize and bind one or more epitopes of the osteoactivin proteins or polypeptide fragments thereof. In one embodiment of this aspect, the invention provides a substantially pure antibody that specifically binds to one or more epitopes of an osteoactivin protein, or a polypeptide fragment thereof, wherein said osteoactivin protein stimulates bone cell differentiation. The invention further provides a substantially pure antibody that specifically binds to one or more epitopes of an osteoactivin protein, or a polypeptide fragment thereof, wherein said osteoactivin protein comprises the amino acid sequence of SEQ ID NO:6. In a related embodiment, a substantially pure antibody that specifically binds to one or more epitopes of an osteoactivin protein, or polypeptide fragment thereof, wherein said osteoactivin protein comprises the amino acid sequence of SEQ ID NO:2 is provided. In a preferred embodiment, the antibody is selected from the group consisting of an antibody which binds to one or more epitopes of an osteoactivin peptide 35 having SEQ ID NO:3 and an antibody which binds to one or more epitopes of an osteoactivin peptide 551 having SEQ ID NO:4. In a particularly preferred embodiment, the antibody binds to one or more epitopes of amino acids 538-553 of SEQ ID NO:6. Another preferred embodiment is an antibody which specifically binds to an

osteoactivin protein, or polypeptide fragment thereof, having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2, wherein said osteoactivin protein or polypeptide fragment thereof stimulates bone cell differentiation or bone formation. In each of these embodiments, the antibody may be a polyclonal or a monoclonal antibody.

The term "antibody" as used herein refers to an immunoglobulin molecule or immunologically active portion thereof, *i.e.*, an antigen-binding portion. Examples of immunologically active portions of immunoglobulin molecules include F(ab), Fv, and F(ab')₂ fragments which can be generated by cleaving the antibody with an enzyme such as pepsin.

The term "epitope" as used herein means that region of amino acid residues of an osteoactivin protein antigen that is specifically recognized by an anti-osteoactivin antibody.

By "specifically binds" means an antibody that physically interacts with its specific ligand (*i.e.*, an osteoactivin protein or biologically active polypeptide fragment thereof) with greater affinity that it binds to other molecules.

The invention further provides methods for producing a substantially pure osteoactivin protein, or polypeptide fragment thereof, comprising: (a) culturing a cell stably transformed with a gene comprising a nucleic acid molecule encoding an osteoactivin protein, wherein said nucleic acid comprises the nucleic acid sequence from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1; and (b) isolating and purifying the osteoactivin protein from the culture medium. Another preferred embodiment includes a method for producing a substantially pure osteoactivin protein, or polypeptide fragment thereof, comprising: (a) culturing a cell stably transformed with a gene comprising said nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1; and (b) isolating and purifying said osteoactivin protein from said culture medium. A method for producing a substantially pure osteoactivin protein, or polypeptide fragment thereof, comprising: (a) culturing a cell stably transfected with a vector comprising the nucleic acid molecule encoding an osteoactivin protein, wherein said nucleic acid comprises the nucleic acid sequence from nucleotide 115 to

nucleotide 1,830 of SEQ ID NO:1; and (b) isolating and purifying said osteoactivin protein from said culture medium, is also provided. In a related embodiment, the invention provides a method for producing a substantially pure osteoactivin protein, or polypeptide fragment thereof, comprising: (a) culturing a cell stably transfected with a vector comprising said nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence from nucleotide 115 to nucleotide 1,830 of SEQ ID NO:1; and (b) isolating and purifying said osteoactivin protein from said culture medium.

As used herein, the terms osteoactivin "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding an osteoactivin protein, such as a mammalian osteoactivin protein, and can further include non-coding regulatory sequences, and introns. These genes can be isolated from genomic DNA, cloned by recombinant means, or chemically synthesized.

As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a prokaryotic or eukaryotic host cell, including, but not limited to, calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation, such that the DNA within the vector is expressed in the host cell.

The invention also provides for therapeutic compositions of the disclosed nucleic acid molecules and osteoactivin proteins and antibodies. Accordingly, in another aspect, the invention provides a therapeutic composition comprising a pharmaceutically acceptable carrier or delivery vehicle and a nucleic acid encoding an osteoactivin protein, or biologically active polypeptide fragment thereof, wherein said osteoactivin protein stimulates bone cell differentiation. In some embodiments, the therapeutic composition comprises a nucleic acid molecule encoding a human osteoactivin or encoding the amino acid sequence of SEQ ID NO:6. In still another embodiment, the invention encompasses a therapeutic composition comprising a nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence of SEQ ID NO:1, and a pharmaceutically acceptable delivery vehicle.

In certain embodiments of this aspect of the invention, the therapeutic composition further comprises a mediator such as a cytokine or a growth factor. Non-limiting examples of such mediators include interleukin-1, tumor necrosis factor, lymphotoxin, interleukin-6, prostaglandins of the E-series, leukotrienes, lipopolysaccharides, transforming growth factor- β , and colony-stimulating factors. In another related aspect, the invention provides a therapeutic composition comprising an agent that stimulates osteoactivin-mediated bone formation. The term "mediator" refers to a molecule that directly modulates, mediates, or changes the expression of an osteoactivin protein.

As used herein, a "therapeutic composition" refers to a composition comprising an active ingredient required to cause a desired effect when a therapeutically effective amount of the composition is administered to a mammal in need thereof.

Within the present invention, a "therapeutically effective amount" of a composition is that amount of each active component of the therapeutic composition that is sufficient to show a benefit (*e.g.*, a reduction in a symptom associated with the disorder, disease, or condition being treated). When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the benefit, whether administered in combination, serially, or simultaneously.

As used herein, the term "pharmaceutically acceptable delivery vehicle" refers to carriers that do not negatively affect the biological activity of the therapeutic molecule or compound to be placed therein. Preferably, the vehicle targets bone cells. The characteristics of the delivery vehicle will depend on the route of administration. Therapeutic compositions may contain, in addition to the active compound, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art.

Other therapeutic compositions within the scope of this invention cover compositions comprising vectors. Accordingly, in one embodiment, the invention features a therapeutic composition comprising a biologically functional expression vector comprising a nucleic acid sequence encoding an osteoactivin protein,

wherein said osteoactivin protein stimulates bone cell differentiation or bone formation, and a pharmaceutically acceptable delivery vehicle. In another embodiment, the invention provides a therapeutic composition comprising a biologically functional expression vector comprising a nucleic acid molecule encoding an osteoactivin protein having at least 92% sequence identity with the nucleic acid sequence of SEQ ID NO:1, and a pharmaceutically acceptable delivery vehicle. In certain embodiments of this aspect of the invention, the therapeutic composition further comprises a mediator, such as interleukin-1, tumor necrosis factor, lymphotoxin, interleukin-6, prostaglandins of the E-series, leukotrienes, lipopolysaccharides, transforming growth factor- β , or colony-stimulating factors, or a nucleic acid molecule encoding any of these mediator polypeptides.

Still other embodiments within this aspect of the invention are directed to therapeutic compositions comprising an osteoactivin protein. Accordingly, the invention features a therapeutic composition comprising a pharmaceutically acceptable carrier or delivery vehicle and an osteoactivin protein, or biologically active polypeptide fragment thereof, wherein said osteoactivin protein stimulates bone cell differentiation or bone formation. In some embodiments, the osteoactivin protein in the therapeutic composition is human. In other embodiments, the osteoactivin protein comprises SEQ ID NO:6. In another embodiment of this aspect, the invention covers a therapeutic composition comprising an osteoactivin protein, or polypeptide fragment thereof, wherein said protein comprises the amino acid sequence of SEQ ID NO:2, and a pharmaceutically acceptable delivery vehicle. In yet a further embodiment, a therapeutic composition comprising a pharmaceutically acceptable delivery vehicle and a non-human, non-murine osteoactivin protein, or polypeptide fragment thereof, having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2, wherein said osteoactivin protein or polypeptide fragment thereof stimulates bone cell differentiation or bone formation. In certain embodiments of this aspect of the invention, the therapeutic composition may further comprises a mediator, including interleukin-1, tumor necrosis factor, lymphotoxin, interleukin-6, prostaglandins of the E-series, leukotrienes, lipopolysaccharides, transforming growth factor- β , and colony-stimulating factors.

In another aspect, the invention provides a therapeutic composition comprising an agent that inhibits osteoactivin-mediated bone formation, and a pharmaceutically acceptable delivery vehicle.

As used herein, a "biologically active portion" of an osteoactivin protein includes a fragment of an osteoactivin protein which is capable of affecting bone differentiation or bone formation.

Additional therapeutic compositions of the invention relate to those comprising antibodies that react with, or specifically bind, osteoactivin proteins. In one embodiment of this aspect, the invention provides a therapeutic composition comprising a pharmaceutically acceptable delivery vehicle and an antibody that specifically binds to one or more epitopes of an osteoactivin protein, or polypeptide fragment thereof, wherein said osteoactivin protein stimulates bone differentiation or bone formation. In a related embodiment, the invention covers a therapeutic composition comprising a substantially pure antibody that specifically binds to one or more epitopes of an osteoactivin protein, or polypeptide fragment thereof, wherein said osteoactivin protein comprises the amino acid sequence of SEQ ID NO:2, and a pharmaceutically acceptable delivery vehicle. In a preferred embodiment, the antibody of a therapeutic composition is selected from the group consisting of an antibody which binds to one or more epitopes of an osteoactivin peptide 35 having SEQ ID NO:3, an antibody which binds to one or more epitopes of an osteoactivin peptide 551 having SEQ ID NO:4, and an antibody which binds to one or more epitopes of amino acids 538-553 of the human osteoactivin protein of SEQ ID NO:6, together with a pharmaceutically acceptable delivery vehicle. Another preferred antibody, according to this aspect of the invention, is one in which the antibody of the therapeutic composition specifically binds to a non-human, non-murine osteoactivin protein, or polypeptide fragment thereof, having at least 90% sequence identity with the amino acid sequence of SEQ ID NO:2, wherein said osteoactivin protein or polypeptide fragment thereof stimulates bone cell differentiation or bone formation, together with a pharmaceutically acceptable delivery vehicle. In certain embodiments, the antibody of the invention is a polyclonal or a monoclonal antibody.

In yet another aspect, the invention provides *in vivo* methods of stimulating bone formation in a mammal, comprising administering to said mammal a therapeutically effective amount of a nucleic acid molecule encoding an osteoactivin protein or peptide thereof, or an osteoactivin protein, or biologically active polypeptide fragment thereof, or an agent that enhances osteoactivin-mediated bone cell differentiation or bone formation. In other embodiments of this aspect, *ex vivo* methods for stimulating bone formation in a human are described, comprising the steps of: (a) extracting osteoblast cells from said human; (b) contacting said osteoblast cells with a therapeutically effective amount of a nucleic acid molecule encoding an osteoactivin protein, or an osteoactivin protein, or biologically active polypeptide fragment thereof; and (c) returning said cells to the bone of said human.

"Mammal" as used herein means any animal classified as a mammal including humans, cows, horses, dogs, mice, cats, goats, pigs, and sheep.

In another aspect, the invention features *in vivo* methods for inhibiting bone formation in a mammal, comprising administering to said mammal a therapeutically effective amount of any of the therapeutic compositions of the present invention comprising antibodies. In a related aspect, the invention also provides a method for inhibiting bone formation in a mammal, comprising administering to said mammal a therapeutically effective amount of an agent that inhibits osteoactivin-mediated bone formation.

In another aspect, the invention provides *in vivo* methods of inhibiting bone formation or bone cell differentiation in a mammal, comprising administering to said mammal a therapeutically effective amount of any of the therapeutic compositions of the invention comprising, in part, an antibody.

The invention provides, in yet another aspect, *in vivo* methods of treating bone disorders in a mammal, such as a human, comprising administering to said mammal a therapeutically effective amount of any of the therapeutic compositions of the invention, comprising an anti-osteoactivin antibody or an agent that inhibits osteoactivin-mediated bone differentiation. In a related embodiment, the invention provides *ex vivo* methods for treating bone disorders in a mammal, comprising the steps of: (a) extracting osteoblast cells from said mammal;

(b) contacting said osteoblast cells with a therapeutically effective amount of an antibody specific for osteoactivin protein or an agent that inhibits osteoactivin-mediated bone cell differentiation or bone formation; and (c) returning said contacted cells to the bone of said mammal. In preferred embodiments, the bone disorder treated by the method is selected from the group consisting of an ectopic bone formation, osteoporosis, periodontal disease, and osteopetrosis.

The phrase "bone disorder," as used herein, refers to a pathological disorder, disease, or condition in a mammal in which there is an imbalance in the ratio of bone formation to bone resorption, such that, if left untreated, would result in that mammal exhibiting an abnormal mass of bone.

"Treating," "treatment," and "therapy," as used herein, refer to curative, prophylactic, or preventative manipulations, or manipulations which stimulate bone cell differentiation or bone formation, postpone the development of bone disorder symptoms, and/or reduce the severity of bone disorders and/or such symptoms that will or are expected to develop from a bone disorder. The terms further include ameliorating existing bone disorder symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, preventing or reversing metabolic causes of symptoms, preventing or reversing bone growth, and/or encouraging bone resorption. Thus, the terms denote that a beneficial result has been conferred on a mammal with a bone disorder, or with the potential to develop such disorder.

In another aspect, the invention provides *in vivo* methods of treating bone disorders in a mammal such as a human, comprising administering to said mammal, a therapeutically effective amount of any of therapeutic compositions of the invention comprising a nucleic acid molecule encoding an osteoactivin protein, or an osteoactivin protein, or biologically active polypeptide fragment thereof, wherein said osteoactivin protein or biologically active polypeptide fragment thereof stimulates bone formation or bone cell differentiation. In other embodiments of this aspect, *ex vivo* methods for treating bone disorders in a mammal are provided. These methods comprise the steps of: (a) extracting osteoblast cells from said mammal; (b) contacting said osteoblast cells with a therapeutically effective amount of any of the therapeutic compositions

comprising a nucleic acid molecule encoding an osteoactivin protein, or biologically active polypeptide fragment thereof, of the invention; and (c) returning said cells to the bone of said mammal, wherein said osteoactivin protein or biologically active polypeptide fragment thereof stimulates bone formation or bone cell differentiation. In certain embodiments, the bone disorder treated is selected from the group consisting of an ectopic bone formation, osteoporosis, periodontal disease, and osteopetrosis. In other certain embodiments of this aspect, the method results in inhibition of bone resorption.

In yet another aspect, the invention provides methods for identifying an agent that modulates the expression or activity of osteoactivin nucleic acid molecules or proteins.

As used herein, an "agent" is a candidate or test compound (*e.g.*, a protein, peptide, peptidomimetic, peptoid, small molecule or other chemical entity) which modulates the expression or activity of the osteoactivin nucleic acid molecule or protein. By "modulating the expression or activity of the osteoactivin nucleic acid molecule or protein" is meant a compound or molecule that has a stimulatory or inhibitory effect on, for example, osteoactivin expression or osteoactivin activity.

The method uses cells capable of expressing a gene under the control of the regulatory element(s) of an osteoactivin gene. Such cells include those which are capable of expressing an endogenous osteoactivin gene (*e.g.*, an osteoblast cell line) or a cell transfected with a transgene comprising an osteoactivin regulatory element (*e.g.*, an osteoactivin promoter) fused to a nucleic acid sequence encoding a polypeptide (*e.g.*, an osteoactivin protein or a reporter protein), such that the osteoactivin gene regulatory element controls expression of the coding sequence.

In a preferred embodiment, the method uses host cells transfected with a nucleic acid comprising an osteoactivin regulatory element fused with nucleic acid sequence encoding a reporter protein. The preferred osteoactivin regulatory element comprises sequences spanning from just upstream of the ATG start site to 8-10 kb upstream of the ATG start site. The method comprises culturing separate samples of cells in the presence and absence of an agent in a suitable culture medium, wherein said cells express a gene under the control of an osteoactivin

regulatory element; and measuring and comparing the levels of expression of said gene from said samples of cells cultured in the presence and absence of agent.

In still another aspect, the invention provides assays for determining the presence or absence of a genetic alteration in an osteoactivin polypeptide or in a nucleic acid encoding an osteoactivin protein. One embodiment of the invention is an assay for diagnosing osteopetrosis in a mammal suspected of suffering from osteopetrosis, comprising: (a) measuring the level of osteoactivin protein expression in a biological sample from said mammal; and (b) comparing said level of osteoactivin protein expression to a level of osteoactivin protein expression in a biological sample from a control.

In another aspect, a method for diagnosis of osteopetrosis in a mammal is provided. In this method, the level of osteoactivin in the mammal is measured and compared with the level of osteoactivin expressed in a control mammal which does not suffer from osteopetrosis, wherein increased expression in (a) compared to (b) is indicative of osteopetrosis in the mammal in (a).

BRIEF DESCRIPTION OF THE DRAWINGS

Sub 1 > Figure 1A is a schematic representation of the nucleotide sequence and corresponding amino acid sequence of rat osteoactivin and its predicted amino acid sequence (beginning with the methionine at nucleotide 115) shown in single letter format below the DNA sequence. Solid black lines between nucleotides 217 to 267 and 1768 to 1818 underline the peptides to which the antisera were raised for immunohistochemical localization and Western blot analysis of osteoactivin expression.

Figure 1B is a chart characterizing the structure of the human osteoactivin gene from BAC clone RG271G13.

Figure 1C is a graphic representation of the results of hydropathy analysis of osteoactivin.

Sub 2 > Figure 2A is a schematic representation of the alignments of the open reading frame nucleotide sequences of rat osteoactivin, mouse *nmb*, and human *nmb*.

Figure 2B is a schematic representation of the alignment of the predicted amino acid sequences of rat osteoactivin, mouse *nmb*, and human *nmb*.

Figure 3 is a representation of an autoradiograph of a differential display gel showing osteoactivin (arrow) in mutant (M) and normal (N) long bone (L) and calvaria (C).

Figure 4A is a representation of a Northern blot showing osteoactivin expression in the mutant calvaria (M) which was 5-to 7-fold higher than in the normal calvaria (N). Similar results were obtained when RNA from mutant (M) and normal (N) long bone were compared.

Figure 4B is a representation of the same Northern blot shown in Figure 4A after stripping and reprobing with a probe for 18s rRNA.

Figure 5 is a representation showing the immunolocalization of osteoactivin in primary rat osteoblasts, wherein immunofluorescent staining was primarily observed in the perinuclear region of the cell, consistent with localization in the secretory pathways of the cell.

Figure 5A is a representation of an electron micrograph showing the immunolocalization of osteoactivin in primary rat osteoblasts, wherein

immunofluorescent staining was primarily observed in the perinuclear region of the cell, consistent with localization in the secretory pathways of the cell. Primary rat osteoblasts cultured for 5 days were fixed and incubated with chicken anti-osteocalcin primary antibody followed by incubation with a Cy3-conjugated secondary antibody (red). Magnification 175x; insert magnification 350x.

Figure 5B is a representation of an electron micrograph showing the immunolocalization of the rough endoplasmic reticular (RER) in primary rat osteoblasts. Cells were then stained to visualize the RER using DiOC₅ dye (green). Magnification 175x; insert magnification 350x.

Figure 5C is a representation of an electron micrograph showing the co-localization of immunofluorescent staining of osteocalcin with the immunofluorescent staining of the rough endoplasmic reticulum (RER). Magnification 175x; insert magnification 350x. Images of Figure 5A and Figure 5B were overlaid to demonstrate co-localization of staining for osteocalcin with the RER (yellow).

Figure 6 is a representation of a Northern blot of osteocalcin expression in calvaria and long bone of mutant (M) and normal (N) rats 2 weeks (2 wk), 4 weeks (4 wk) and 6 weeks (6 wk) old. Osteocalcin was expressed at higher levels in the mutant bones at all ages examined, and appeared to decrease with age in the normal rats, while in the mutants expression remained high, especially in the long bone RNA.

Figure 7A is a representation of a Northern blot showing osteocalcin expression in primary rat osteoblast cells derived from normal (N) or mutant (M) calvaria cultured for 1 week, 2 weeks, or 3 weeks.

Figure 7B is a representation of the same Northern blot in Figure 7A which was stripped and reprobed with a probe for 18S rRNA.

Figure 8 is a representation of a Western blot showing secreted osteocalcin protein from osteoblasts isolated and cultured for one week from either mutant (M) or normal (N) rat calvaria and probed with a chicken anti-rat osteocalcin antibody raised against peptide 551 (SEQ ID NO:4).

Figure 9 is a graphic representation of the quantitation of a Western blot of osteocalcin expression (normalized as to β -tubulin as a control for protein loading

and blotting efficiency) in long bones from 2 week (2 wk), 4 week (4 wk), and 6 week (6 wk) old mutant (M) or normal (N) rats.

Figure 10 is a graphic representation of the ability of anti-osteoactivin antibodies to inhibit osteoblast differentiation as measured by calcium deposition.

FIGURE 10

DETAILED DESCRIPTION OF THE INVENTION

The patent applications, patents, and literature references cited herein indicate the knowledge of those of ordinary skill in this field and are hereby incorporated by reference in their entirety. In the case of inconsistencies between any reference cited herein and the specific teachings of the present disclosure, this disclosure will prevail. Similarly, any inconsistencies between an art-understood meaning of a term and a meaning of a term as specifically taught in the present disclosure will be resolved in favor of this disclosure.

The present invention discloses a cDNA encoding a novel protein, osteoactivin, first identified in the bone of rats carrying the *op* mutation. A comparison of gene expression in normal versus *op* long bones and calvaria using mRNA-differential display resulted in the identification and cloning of a cDNA encoding the osteoactivin protein. Osteoactivin mRNA is highly over-expressed in *op* versus normal bone. These findings provide evidence that the protein encoded by this cDNA plays a role in osteoblast development, bone cell differentiation, and bone formation, and therefore, is involved in normal skeletal modeling/remodeling.

Nucleic Acid Molecules Encoding an Osteoactivin Protein

The rat full-length osteoactivin nucleic acid sequence (Figure 1A; SEQ ID NO:1), which is approximately 2320 nucleotides long including untranslated regions, contains a predicted methionine-initiated coding sequence of 1716 nucleotides (nucleotides 115-1830 of SEQ ID NO:1). This full-length nucleic acid sequence has been deposited in GenBank and has Accession Number AF184983.

A GenBank search identified human and mouse *nmb* proteins found in melanoma cell lines that are likely homologs of the rat osteoactivin protein disclosed herein (Waterman *et al.*, *Int. J. Cancer* 60:73-81 (1995); Bachner *et al.*, GenBank Accession No. AJ 251685). A comparison of the nucleotide sequences of the open reading frames of the rat osteoactivin, human *nmb*, and mouse *nmb*, genes is shown in Figure 2A. Of the 1716 nucleotides in the rat osteoactivin coding region, 1304 nucleotides were identical in human, which corresponds to 76%

sequence identity. Of the 1716 nucleotides in the rat osteoactivin coding region, 1574 nucleotides were identical in mouse, which corresponds to 91% sequence identity.

To determine the percent sequence identity of two amino acid sequences, or of two nucleic acid sequences, the sequences were aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). Preferably, the length of a reference sequence aligned for comparison purposes is at least 30%, more preferably at least 40%, even more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions were then compared. When a position in the first sequence was occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, the molecules were considered identical at that position. The percent sequence identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

molecule is within a sequence identity or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5. The percent sequence identity between two amino acid or nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller (*CABIOS*, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (*J. Mol. Biol.* **215**:403-10 (1990)). BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to osteoactivin nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to osteoactivin protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (*Nucleic Acids Res.* **25**(17):3389-3402 (1997)). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (*see*, <http://www.ncbi.nlm.nih.gov>).

tissues and that this site mapped to the end of the human nmb cDNA as previously reported. Osteoactivin is expressed in human osteoblasts in culture as a single transcript of approximately 2.4 kb.

The invention further contemplates nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1, or the nucleotide sequence of the DNA insert of the plasmid deposited with GenBank as Accession Number AF184983. Such differences can be due to degeneracy of the genetic code, and result in a nucleic acid which encodes the same osteoactivin proteins as those encoded by the nucleotide sequence disclosed herein. The invention provides an isolated nucleic acid molecule encoding a protein having an amino acid sequence which differs by at least 1, but by less than 5, 10, 20, 50, or 100 amino acid residues than shown in SEQ ID NO:2. If alignment is needed for this comparison, the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.

Nucleic acid molecules of the invention can be chosen for having codons which are preferred or non-preferred for a particular expression system. For example, the nucleic acid can be one in which at least one codon, and preferably at least 10%, or 20% of the codons, have been altered such that the sequence is optimized for expression in, *e.g.*, *E. coli*, yeast, human, insect, or Chinese hamster ovary (CHO) cells.

Nucleic acid variants can be naturally occurring, such as allelic variants (same locus), homologs (different locus), and orthologs (different organism), or can be non-naturally occurring. Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product), as described below.

Preferably, the nucleic acid sequence differs from that of SEQ ID NO:1, or the sequence in GenBank Accession Number AF184983, *e.g.*, by at least one nucleotide but less than 10, 20, 30, or 40 nucleotides. Alternatively, the nucleic acid sequence differs from that of SEQ ID NO:1 or of AF18493 by at least one but less than 1%, 5%,

10% or 20% of the nucleotides in the subject nucleic acid. If necessary for this analysis the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.

SignalP analysis (CBS SignalP V1.1 World Wide Web Prediction Server at the Center for Biological Sequence (<http://www.cbs.dtu.dk>)) was used to analyze the sequence of osteoactivin for the presence of a signal peptide. Signal P calculates the maximal C- (raw cleavage site score), S-(signal peptide score), and Y-(combined cleavage site score) scores, and the mean S-score, between the N-terminal and the predicted cleavage site. SignalP analysis of osteoactivin revealed a mean S score of 0.907, indicating that osteoactivin has a signal sequence. The cleavage site for the signal peptide was predicted to occur between residues 22 and 23 of rat osteoactivin (SEQ ID NO:2). This region is conserved across species, indicating that mouse and human osteoactivin contain the same leader sequence and cleavage site. Accordingly, osteoactivin polypeptides lacking the signal sequence are functionally active osteoactivin polypeptides.

The invention, therefore, provides for nucleic acid molecules encoding the osteoactivin polypeptide lacking the signal sequence, wherein the osteoactivin polypeptide comprises amino acid residues 23-572 of SEQ ID NO:2. This nucleic acid molecule encoding the osteoactivin protein lacking the signal sequences comprises the nucleic acid sequence of nucleotides 181-1830 of SEQ ID NO:1.

Orthologs, homologs, and allelic variants can be identified using methods known in the art. These variants comprise a nucleotide sequence encoding a polypeptide that is 50%, at least 55%, typically at least 70-75%, more typically at least 80-85%, and most typically at least 90-95% or more identical to the nucleotide sequence shown in SEQ ID NO:1 or to a fragment of this sequence. Such nucleic acid molecules can readily be identified as being able to hybridize under moderately stringent conditions to the nucleotide sequence shown in SEQ ID NO:1, or a fragment of the sequence. Nucleic acid molecules corresponding to orthologs, homologs, and allelic variants of the osteoactivin cDNAs of the invention can further be isolated by mapping to the same chromosome or locus as the osteoactivin gene. Preferred variants are those that maintain their biological function including the ability to bind osteoactivin binding partners.

An “allelic variant,” as used herein, is a protein having at least 75% identity, preferably at least 85%, more preferably at least 95%, and most preferably at least 99% identity to the amino acid sequence of osteoactivin, or to a fragment thereof, or to a protein conjugate thereof which retains the biological activity of osteoactivin. Allelic variants of osteoactivin include both functional and non-functional proteins. Functional allelic variants are naturally occurring amino acid sequence variants of the osteoactivin protein within a population that maintain their biological function including the ability to bind osteoactivin binding partners. Functional allelic variants will typically contain only conservative substitution(s) of one or more amino acids of SEQ ID NO:2, or the substitution, deletion or insertion of non-critical residues in non-critical regions of the protein. Non-functional allelic variants are naturally-occurring amino acid sequence variants of the osteoactivin protein, *e.g.*, human osteoactivin protein, within a population that does not have osteoactivin biological activity such as the ability to bind osteoactivin binding partners. Non-functional allelic variants will typically contain a non-conservative substitution, a deletion or insertion, or premature truncation of the amino acid sequence of SEQ ID NO:2, or a substitution, insertion, or deletion in critical residues or critical regions of the protein.

Osteoactivin Proteins and Polypeptide Fragments

The nucleotide coding sequence encodes a 572 amino acid protein shown in Figure 1A (SEQ ID NO:2). The protein has a predicted molecular weight of 63.8 kD. Hydropathy analysis, shown in Figure 1C, reveals a potential leader sequence with a cleavage site after amino acid residue 22 in SEQ ID NO:2, as well as several potential transmembrane spanning regions throughout the molecule.

Figures 2B shows the amino acid sequences of rat osteoactivin, human nmb, and mouse nmb, aligned to determine the percentage of sequence identity. Of the 572 amino acid residues in the rat osteoactivin protein, 394 amino acid residues were identical in human, which corresponds to 69% sequence identity. Notably, the predicted protein sequence of the rat osteoactivin has a proline/serine-rich 14 amino acid residue insertion beginning at amino acid residue 33 that is not present in the human nmb homolog. Of the 572 amino acid

residues in the rat osteoactivin protein, 509 amino acid residues were identical to mouse, which corresponds to 89% sequence identity.

An osteoactivin protein of the invention is a protein which comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5 or SEQ ID NO:6, and alternatively or additionally, comprises an osteoactivin protein having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5 or SEQ ID NO:6 and stimulates bone cell differentiation or bone formation. An osteoactivin protein of the invention further comprises the osteoactivin protein sequence lacking the signal sequence comprising amino acid residues 23-572 of SEQ ID NO:2.

The osteoactivin proteins, polypeptide fragments thereof, mutants, truncations, derivatives, and splice variants of SEQ ID NO:2 that display substantially equivalent or altered osteoactivin activity relative to SEQ ID NO:2 are likewise contemplated. These variants may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the osteoactivin protein. Included within the scope of these terms are osteoactivin proteins specifically recited herein, as well as all substantially homologous analogs and allelic variants.

Analogous may be made through substitution of conserved amino acids. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted non-essential amino acid residue in an osteoactivin protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can

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The biologically functional recombinant expression vectors of the invention can be designed for expression of osteoactivin proteins in prokaryotic or eukaryotic cells. For example, representative osteoactivin expression vectors are yeast expression vectors and vectors for expression in insect cells (*e.g.*, a baculovirus expression vector) or a vector suitable for expression in mammalian cells such as yeast or CHO cells. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example, using T7 promoter regulatory sequences and T7 polymerase. Suitable host cells are discussed further in Goeddel (*Meth. Enzymol.* **185**:3-7 (1990)).

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enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes and their cognate recognition sequences include, but are not limited to, Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Amersham Pharmacia Biotech Inc., Piscataway, NJ; Smith *et al.*, *Gene* 67:31-40 (1988)), and pMAL (New England Biolabs, Beverly, MA) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A to the target recombinant protein.

Purified fusion proteins can be used in osteoactivin activity assays, (*e.g.*, direct assays or competitive assays described in detail below), or to generate antibodies specific for osteoactivin proteins. In one non-limiting example, a retroviral expression vector encoding a fusion protein can be used to infect bone marrow cells which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (*e.g.*, six weeks).

To maximize recombinant protein expression in *E. coli*, the protein can be expressed in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Meth. Enzymol.* 185:119-128, (1990)). Another strategy is to alter the nucleotide sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, *Nucleic Acids Res.* 20:2111-2118 (1992)). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

When used in mammalian cells, the control functions of the expression vector are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus, or Simian Virus 40.

Antibodies

The present invention also encompasses antibodies that recognize and bind to the osteoactivin protein or fragment thereof (*e.g.*, to one or more epitopes of the protein having SEQ ID NOS:2 or 6). These antibodies are polyclonal or

monoclonal antibodies which may be provided using standard methods (see, *e.g.*, Ausubel *et al.*, *supra*; Coligan, J.E. *et al.*, Current Protocols in Immunology, John Wiley & Sons, New York (1991); and Delves, P.J., Antibody Production: Essential Techniques, John Wiley & Sons, New York (1997)). Briefly, osteoactivin protein or a polypeptide fragment purified according to the methods described for an aspect of the invention are used to immunize rabbits (*e.g.*, for polyclonal antibodies) or mice (*e.g.*, for monoclonal antibodies) to generate antibody-mediated immunity to the osteoactivin protein or polypeptide fragment used to immunize the animal. Monoclonal antibodies can be screened by, *e.g.*, ELISA, to identify those that show the highest affinity for the immunizing osteoactivin protein or polypeptide fragment. The cloned cell producing the high affinity monoclonal antibody can then be propagated *in vitro* (where the antibody is purified from the culture supernatant) or *in vivo* (where the antibody is purified from ascites fluid), and can also be cryopreserved and stored frozen, *e.g.*, at -70°C in DMSO, to provide a potentially limitless supply of monoclonal antibody. Antibodies can also be provided by known recombinant DNA techniques.

In addition to intact monoclonal and polyclonal antibodies, the invention also provides various fragments of an osteoactivin antibody, such as Fab, F(ab')₂, Fv, and sFv fragments, which can be produced by proteolytic cleavage or recombinant DNA techniques. Humanized antibodies are also provided and can be produced according to methods known in the art (see, *e.g.*, Green *et al.*, *Nature Genetics* 7:13-21 (1994)).

Also provided by the invention are osteoactivin protein-specific single polypeptide chain antibodies (see general methods in U.S. Patent Nos. 4,946,788 and 4,704,692); single domain antibodies (see, *e.g.*, Ward *et al.*, *Nature* 341:544-546 (1989)); and chimeric antibodies (see, *e.g.*, U.S. Patent No. 4,816,567). A single-chain antibody (scFV) may be engineered by known methods (see, *e.g.*, Colcher *et al.*, *Ann. NY Acad. Sci.* 880:263-80 (1999); and Reiter, *Clin. Cancer Res.* 2:245-52 (1996)). The single chain antibody can be dimerized or multimerized to generate multivalent antibodies having specificities for different epitopes of the same target osteoactivin protein.

[illegible]

Exemplary preferred epitopes encompassed by the antigenic peptide are regions of osteoactivin located on the surface of the protein, *e.g.*, hydrophilic regions. For example, an Emini surface probability analysis of the human osteoactivin protein sequence can be used to indicate the regions that have a particularly high probability of being localized to the surface of the osteoactivin protein, and are thus likely to constitute surface residues useful for targeting antibody production. However, antibodies of the invention bind an epitope on any domain or region on osteoactivin proteins described herein.

Chimeric, humanized, but most preferably, completely human antibodies are desirable for applications which include repeated administration, *e.g.*, therapeutic treatment (and some diagnostic applications) of human patients.

Some antibodies of the invention have a reduced ability or no ability to bind an Fc receptor, for example, where it is an isotype or subtype, fragment, or other mutant, which does not support binding to an Fc receptor, or where it has a mutagenized or deleted Fc receptor binding region.

An anti-osteostatin antibody (*e.g.*, monoclonal antibody) can be used to isolate osteostatin by standard techniques, such as by affinity chromatography or immunoprecipitation. Moreover, an anti-osteostatin antibody can be used to detect osteostatin protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the protein. Anti-osteostatin antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Non-limiting examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Non-limiting examples of suitable enzyme labels include horseradish peroxidase, alkaline phosphatase, galactosidase, and acetylcholinesterase. Non-limiting examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin. Non-limiting examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, and phycoerythrin. An example of a luminescent material includes, but is not limited to, luminol. Non-limiting examples of bioluminescent materials include luciferase, luciferin, and aequorin. Non-limiting examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , and ^3H . Coupling of labels to antibodies can be accomplished using standard techniques (see, *e.g.*, Antibodies, A Laboratory Manual, Harlow and Lane, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999).

Therapeutic Compositions

The osteoactivin-encoding nucleic acid and osteoactivin polypeptides and fragments thereof, as well as anti-osteoactivin antibodies (also collectively referred to herein as "active compounds"), of the invention, or an agent that modulates osteoactivin activity or expression, can be incorporated into therapeutic compositions. Such compositions typically include osteoactivin nucleic acid molecules, proteins, antibodies, or agents and preferably includes a pharmaceutically acceptable delivery vehicle or carrier. As used herein the language "pharmaceutically acceptable delivery vehicle" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. This delivery vehicle may be targeted to the bone or bone cells by virtue of its composition, for example, using a bisphosphonate tetracycline, or calcein. Alternatively, a vehicle may be a polymer or collagen composition that is applied to bone during surgery or by injection at the bone site (see, *e.g.* U.S. Patent Numbers 4,938,763; 5,278,201; 5,324,519; 5,487,897; 5,599,552; 5,702,716; 5,733,950; 5,739,176; 5,744,153; 5,759,563; 5,780,044; 5,945,115; 5,990,194; and 5,631,243). Additional active compounds can also be incorporated into the compositions.

A therapeutic composition is formulated to be compatible with its intended route of administration. Non-limiting examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, by ingestion or inhalation), transdermal (topical), transmucosal, and rectal administration. Oral administration or injection at a bone site is preferred. Solutions or suspensions can be made as described in Remington's Pharmaceutical Sciences, (18th ed., Gennaro, ed., Mack Publishing Co., Easton, PA, (1990)).

Therapeutic efficacy of such active compounds can be determined by standard therapeutic procedures in cell cultures or experimental animals, *e.g.*, for determining the ED50 (the dose therapeutically effective in 50% of the population).

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage may vary depending upon the dosage form employed and the route of administration. For

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The therapeutic compositions may also include other active or inert components. Of particular interest are those mediators that promote bone growth

or infiltration, such as cytokines and growth factors. Non-limiting exemplary mediators for this purpose include interleukin-1, tumor necrosis factor, lymphotoxin, interleukin-6, prostaglandins of the E-series, leukotrienes, lipopolysaccharides, transforming growth factor- β , and colony-stimulating factors. Agents that promote bone growth, such as bone morphogenic proteins, are also useful.

Within the present invention, a “therapeutically effective amount” of a therapeutic composition is that amount which produces a desired effect. For example, a therapeutically effective amount is the amount of the therapeutic composition comprising the active osteoactivin protein compound herein required to provide an effect in reversing the symptoms of the bone disorder. Such therapeutically effective amounts will be determined using routine optimization techniques that are dependent on the particular condition to be treated, the condition of the patient, the route of administration, the formulation, and the judgment of the practitioner and other factors evident to those skilled in the art. The dosage required for the therapeutic compositions of the invention, for example, in osteoporosis where an increase in bone formation is desired, is manifested as a statistically significant difference in bone mass between treatment and control groups. Other measurements of increases in bone formation and/or bone healing may include, for example, tests for breaking strength and tension, breaking strength and torsion, 4-point bending, increased connectivity in bone biopsies and other biomechanical tests well known to those skilled in the art. General guidance for treatment regimens is obtained from experiments carried out in animal models.

Therapeutic Methods

The therapeutic compositions of the present invention may be used to modulate the differentiation of osteoblasts, osteoblast precursors, and mesenchymal cells *in vivo*, *in vitro*, or *ex vivo*, and to modulate bone cell differentiation and bone formation. As used herein, the term “osteoblast precursor” refers to a cell that is committed to a differentiation pathway, but that generally does not express markers or function as a mature, fully differentiated

cell. Such cells include "mesenchymal cells" or "mesenchymal stem cells," which are pluripotent cells that are capable of dividing many times and whose progeny will give rise to skeletal tissues, including cartilage, bone (osteogenic cells), tendon, ligament, marrow stroma and connective tissue. The disclosed therapeutic compositions or methods are useful for stimulating osteogenesis.

In a preferred method, bone cell differentiation and bone formation is stimulated by administering to a mammal a therapeutic composition comprising a nucleic acid molecule encoding an osteoactivin protein, or comprising an osteoactivin protein or biologically active polypeptide fragment thereof, or comprising an agent that stimulates osteoactivin expression or activity. The appropriate agent can be determined based on screening assays described herein. Bone disorders in mammals that may be treated or prevented by administering one of the above-described therapeutic compositions of the invention include those diseases or pathological conditions in which stimulation of bone cell formation is desired, such as with osteoporosis or bone trauma.

As an alternative, bone cell differentiation and bone formation can be inhibited by administering to a mammal a therapeutic composition comprising an osteoactivin antibody, an osteoactivin antisense nucleic acid, or an agent that inhibits osteoactivin expression or activity. Such therapy is used in treating, for example, osteopetrosis. Examples of osteoactivin antibodies include, for example, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and Fab, F(ab')₂ and Fab expression library fragments, scFV molecules, and epitope-binding fragments thereof. An antisense oligonucleotide directed to the osteoactivin gene or mRNA to inhibit its expression is made according to standard techniques. (See, *e.g.*, Agrawal *et al.* Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs, Vol. 20 (1993)). An agent that inhibits osteoactivin activity or expression is identified by screening assays, as described herein.

In another preferred method, hematopoietic osteogenic cells are removed *ex vivo* from the cell population, either before or after contact or stimulation with a disclosed therapeutic composition. Through well-known practices, the osteogenic

cells may be expanded. The expanded osteogenic cells can be infused or reinfused into a mammal in need thereof.

Screening Methods

The invention provides methods (also referred to herein as "screening assays") for identifying modulators of osteoactivin expression and or osteoactivin activity. Such modulators (*i.e.*, candidates, test compounds, agents, proteins, peptides, peptidomimetics, peptoids, small molecules or other chemical entities) stimulate or inhibit osteoactivin expression or osteoactivin activity. Therefore, agents thus identified can be used to regulate bone cell differentiation and bone formation in a therapeutic protocol.

The test compounds used for screening may be selected individually or obtained from a compound library. Such libraries include biological libraries, peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive)(see, *e.g.*, Zuckermann, *J. Med. Chem.* 37:2678-85 (1994)), spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Dis.* 12:145 (1997)).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example, in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. (USA)* 90:6909 (1993); Erb *et al.*, *Proc. Natl. Acad. Sci. (USA)* 91:11422 (1994); Zuckermann *et al.*, *J. Med. Chem.*, 37:2678 (1994); Cho *et al.*, *Science*, 261:1303 (1995); Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059 (1994); Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061 (1994); and in Gallop *et al.*, *J. Med. Chem.* 37:1233 (1994).

Libraries of compounds may be presented in solution (*e.g.*, Houghten, *Biotechniques*, 13:412-421 (1992)), or on beads (Lam, *Nature* 354:82-841 (1991)), on

transfected with a construct in which the promoter was cloned in the opposite orientation, in a cell line transfected with a construct in which the promoter was absent, and in a different cell line which does not express endogenous osteoactivin and which is transfected with the promoter-reporter construct.

Once a bona fide clonal osteoblastic cell line expressing the human osteoactivin promoter-reporter construct is identified, the cell line is expanded and submitted for high throughput screening to identify agents capable of modulating (*e.g.*, increasing or decreasing) the expression of the reporter gene. When reporter expression is greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of osteoactivin expression. Alternatively, when reporter expression is less in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of osteoactivin expression. The level of reporter expression can be determined by methods described herein for detecting the level of reporter osteoactivin mRNA or protein produced by the cell.

In another embodiment, the osteoactivin protein, or biologically active portion thereof, is contacted with a compound known to bind osteoactivin, *e.g.*, an osteoactivin antibody, to form an assay mixture. The assay mixture is then contacted with a test compound, and the ability of the test compound to interact with an osteoactivin protein is determined. Determining the ability of the test compound to interact with an osteoactivin protein includes determining the ability of the test compound to preferentially bind to osteoactivin or biologically active portion thereof, or to modulate the activity of osteoactivin, as compared to the known compound.

Additionally, the invention encompasses diagnostic and prognostic assays. Accordingly, the presence, level, or absence of osteoactivin protein or nucleic acid expression in a biological sample can be evaluated by methods described herein. In one embodiment, the method comprises obtaining a biological sample from a test mammal and contacting the biological sample with a compound or an agent capable of detecting osteoactivin protein or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes osteoactivin protein such that the presence of osteoactivin protein or nucleic acid is detected in the biological sample. The term "biological

In another example, a control sample is contacted with a compound or agent capable of detecting osteoactivin mRNA, or genomic DNA. The presence of osteoactivin mRNA or genomic DNA in the control sample is then compared with the presence or level of osteoactivin mRNA or genomic DNA in the test sample. The control sample is obtained from a normal bone, whereas the test sample can be obtained from a site of aberrant bone growth.

A variety of methods can be used to determine the level of osteoactivin protein expressed. In general, these methods include using an agent that selectively binds to osteoactivin, such as an antibody, to evaluate the level of osteoactivin in the sample. In a preferred embodiment, the antibody bears a detectable label. Useful antibodies include any of those described above.

The detection methods can be used to detect osteoactivin protein in a biological sample *in vitro* as well as *in vivo*. *In vitro* techniques for detection of osteoactivin protein include enzyme linked immunosorbent assays (ELISAs), immunoprecipitations, immunofluorescence, enzyme immunoassay (EIA), radioimmunoassay (RIA), and Western blot analysis. *In vivo* techniques for detection of osteoactivin protein include introducing into a mammal a labeled anti-osteoactivin antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a mammal can be detected by standard imaging techniques.

The invention is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Reagents

All chemicals were of molecular biology grade or higher and were purchased from Sigma (St. Louis, MO) unless otherwise noted. All cell culture media were purchased from Life Technologies (Gaithersburg, MD).

Animals

An inbred colony of *osteopetrotic* (*op*) mutant rats consisting of heterozygous breeders is maintained at Temple University School of Medicine (Philadelphia, PA). Homozygous mutants (*op/op*) are distinguished from normal littermates (+/?) by radiographic analysis between 1 and 3 days after birth by the failure of the mutants to develop bone marrow cavities. Because the genotype of phenotypically normal rats cannot be distinguished, except by breeding experiments, the normal littermates used in this study were of either heterozygous (+/*op*) or homozygous (+/+) normal genotype. All animals were maintained and used according to the principles in the *NIH Guide for the Care and Use of Laboratory Animals* (1985), and guidelines established by the IACUC of Temple University.

EXAMPLE 1

Primary Osteoblast Cultures

Normal diploid osteoblasts were isolated from the calvaria of 1-3 day old *op/op* mutant or normal littermates rats by sequential trypsin/collagenase digestion and plated in 100 mm dishes in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Calabases, CA) at a density of 5×10^5 cells/dish (Owen, *et al.*, *J. Cell. Physiol.* **143**:420-430 (1990)). Media was changed every other day throughout the time course of culture and for media changes after day 6 of culture, MEM α supplemented with 50 μ g/ml ascorbic acid, 2 mM inorganic phosphate, and 10% FBS was used to feed the cells.

EXAMPLE 2

RNA Isolation

Total cellular RNA was isolated from calvaria and long bones (femurs and tibias) harvested from two week old *op/op* mutant rats and their normal littermates.

Prior to freezing, the ends of the long bones were removed at the growth plate and bone marrow was flushed from the shafts of normal bones with saline (4°C) using a 25-gauge needle. Flushing of the bone marrow was only possible in normal rats; *op* mutants had no marrow cavities. Total RNA was prepared from pools of a minimum of six samples per phenotype and bone site (calvaria versus long bone). Total RNA was prepared as described by Thiede *et al.* (*Endocrinology* 135:929-37 (1994)). Briefly, samples were frozen in liquid nitrogen, pulverized on dry ice, and homogenized in 5 M guanidinium isothiocyanate, 72 mM β -mercaptoethanol, and 0.5% sarkosyl. Homogenates were layered over a cesium chloride (CsCl) cushion (5.7 M CsCl and 30 mM sodium acetate (NaAc)), centrifuged at 100,000 \times g overnight, and total RNA recovered by precipitation of the resulting pellets.

RNA was isolated from the rat osteoblast cultures, as well as from liver, spleen, thymus and brain harvested from 2, 4, and 6 week-old *op* rats and their normal littermates using TRIzol® (Life Technologies, Gaithersburg, MD). The RNA concentration of each sample was quantitated by absorbance at 260 nm. The integrity and accuracy of the spectrophotometric measurement of each RNA sample was assessed by electrophoresis of 1 μ g on an ethidium bromide-stained, formaldehyde-agarose minigel.

EXAMPLE 3

Differential Display of mRNA

Prior to differential display, bone RNA samples were treated with DNase I (Roche Molecular Biochemicals, Indianapolis, IN) to eliminate any potential contamination with genomic DNA. The basic principle of mRNA differential

display was first described by Liang and Pardee (1992) *Science* 257:967-971. Briefly, 0.5 µg RNA from each sample (total of 4 independent samples, mutant and normal, calvaria and long bone) was reverse transcribed using each of 12 two-base-anchored oligo-dT primers provided in the Hieroglyph mRNA profile kits (Beckman Coulter Inc., Fullerton, CA) to subdivide the mRNA population. First strand cDNAs were amplified by the polymerase chain reaction (PCR) for 30 cycles using one of 4 upstream arbitrary primers (also provided in the kit) and the same anchoring primers used for first strand synthesis. This resulted in 48 possible primer combinations for each kit (total of 5 kits) and each PCR amplification was run in duplicate from the same first-strand cDNA template. All amplified cDNAs were radiolabeled with ³³P-dATP ([α-³³P]dATP, 2500 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ). The radiolabeled PCR products were electrophoresed on 4.5% denaturing polyacrylamide gels and dried using the Genomix LR differential display apparatus (Beckman Coulter).

As shown in Figure 3, differential display analysis of *op/op* mutant versus normal calvaria and long bone RNA revealed an mRNA that was overexpressed in the *op/op* mutant bone RNA.

Following autoradiography, the bands were visually assessed and those representing differentially expressed cDNAs (exclusively expressed or highly overexpressed in one phenotype and confirmed in duplicate PCR amplifications) were excised from the gel. Each cDNA of interest was reamplified by PCR and used to probe a Northern blot to confirm its differential expression.

EXAMPLE 4

Northern Blot Analysis

Twenty μg of total RNA from *op* mutant and normal bone/soft tissue or normal osteoblast cultures were electrophoresed on 1% formaldehyde-agarose gels and transferred to nylon membranes (Scheicher & Schuell, Keene, NH). Blots were hybridized with a ^{32}P -labeled ($[\alpha\text{-}^{32}\text{P}]\text{dCTP}$, 6000 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) full length rat osteoactivin cDNA probe (RediprimeTMII, Amersham Pharmacia Biotech) using methods described in Thiede *et al.*,

Endocrinology 135:929-37 (1994). Blots were then autoradiographed, stripped and re-probed with an 18s rRNA probe used as a control to normalize for differences in loading and transfer.

Figure 4A shows that the osteoactivin mRNA expression levels were 5-7 times higher in the *op* mutant (M) calvaria as compared to the normal (N) calvaria. cDNAs which were confirmed to be differentially expressed by Northern blot analysis were cloned into PCR-Script (Stratagene, LaJolla, CA), miniprep DNA was prepared, and plasmids with the appropriately sized inserts were sequenced.

EXAMPLE 5 Cloning of Rat Osteoactivin cDNA

Approximately 600 bp of sequence corresponding to the 3' end of rat osteoactivin was obtained from the differential display clone. This fragment was used as a probe to screen a rat kidney cDNA library by conventional means. A single clone was identified after three rounds of screening and DNA sequence analysis showed that it contained an open reading frame of 1719 bp as depicted in Figure 1A. Following confirmation by Northern blot analysis (see Figures 4A and 4B), this clone was sequenced and found to be related to a human sequence (*nmb*) of unknown function. This cDNA and predicted protein was named osteoactivin to reflect its potential role in osteoblast function. Cloning of the entire coding region of osteoactivin nucleic acid sequence revealed an open reading frame capable of encoding a protein of 572 amino acids with 70% identity to human *nmb* as depicted in Figure 2B. Osteoactivin has a predicted molecular weight of 63.8 kD. Hydropathy analysis, as provided in Figure 1B revealed a potential leader sequence and several potential transmembrane spanning domains throughout the protein, suggesting membrane association.

EXAMPLE 6 DNA Sequencing

DNA was sequenced using standard dideoxy methodologies. Gaps and ambiguities in the sequence were handled by direct sequencing of required

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EXAMPLE 7

Antibody Preparation

Peptide 35 H-CPDHMRENNQLRGWSSDE-NH₂ (SEQ ID NO:3) and peptide 551 H-KAPFSRGDREKDPLLQDKC-NH₂ (SEQ ID NO:4) were conjugated to Keyhole Limpet Hemocyanin (KLH) by Cys residues added at N-terminal end of peptide 35 of the osteoactivin protein (SEQ ID NO:2) or at C-terminal end of peptide 551. For each peptide, two chickens were immunized with 100 µg/chicken /Freunds complete adjuvant on the following immunization schedule: Day 1, pre-immune eggs collected, first boost; Day 14, 2nd boost; Day 28, 3rd boost; Day 42, 4th boost; and Day 49, begin collecting eggs. Twelve eggs were collected and pooled from each chicken. Affinity purified peptide-specific immune IgY was purified by affinity chromatography using peptide immobilized on column. (Hanlos and Lane, *supra*).

EXAMPLE 8

Immunolocalization of Osteoactivin in Primary Rat Osteoblasts

Primary osteoblasts isolated from newborn normal rat calvaria were plated in 100 mm dishes at a density of 5×10^5 cells/dish in Earl's medium supplemented with 10% fetal bovine serum (FBS), 50 $\mu\text{g/ml}$ ascorbic acid, and 10 mM β -glycerolphosphate and cultured for 1 week. Cells were fixed in 4% paraformaldehyde for 10 minutes at 4°C , treated with 0.1% Triton X-100 for 10

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As shown in Figure 6, osteoactivin expression was higher in mutant calvaria and long bones when compared to normal bones at all ages examined. In normal animals, osteoactivin expression decreases with age in both calvaria and long bones. However, in the mutants, osteoactivin expression is still detectable in the calvaria at six weeks of age and, in long bones, is highly expressed at all ages examined.

EXAMPLE 10

As shown in Figure 7A, the temporal pattern demonstrated a remarkable increase in expression between 1 week (proliferation stage) and 2 weeks (matrix maturation stage), with a modest decrease at 3 weeks in culture (mineralization stage). There was no significant difference between osteoblast cultures derived from normal or mutant calvaria. The blot was stripped and re-probed for 18s rRNA to normalize for differences in loading and/or blotting (Figure 7B). Similar results were obtained from two separate experiments.

EXAMPLE 11

Detection of Secreted Osteoactivin Protein in Osteoblast Culture Conditioned Media

Osteoblast cultures were established from the calvaria of newborn *op/op* mutant rats or their normal littermates by established methods and plated at a density of 500,000 cells per 100 mm dish in MEM α supplemented with 10% FBS. Cell culture media was changed every other day. Six days after the initiation of the cultures, the cells were washed twice with culture media lacking FBS. 10 ml of serum free culture medium was then added per 100 mm dish and the cells were cultured for an additional 24 hours. This medium was then harvested and the proteins concentrated using a 10 kD molecular weight cut-off concentrator (Millipore, Bedford, MA). The concentration of protein in these concentrated samples was determined by the method of Bradford (*Analyst. Biochem.* 72:246-54 (1976)) using reagents from Pierce (Rockford, IL). 10 μ g of concentrated protein from the *op/op* mutant or normal osteoblast cultures was electrophoresed in a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to polyvinylidene fluoride (PVDF) membrane by electroblotting. This blot was blocked for 1 hour at room temperature in 10% non-fat milk in phosphate buffered saline (PBS)-0.2% Tween 20 (PBS-Tween) and then incubated overnight at 4°C in blocking solution containing 0.325 μ g/ml chicken anti-rat osteoactivin 551 antibody. Following four washes in PBS-Tween, the blot was incubated at RT for 1 hour in blocking solution containing a 1:5000 dilution of horseradish peroxidase conjugated donkey anti-chicken antibody (Jackson ImmunoResearch, West Grove, PA). Following four washes in PBS-Tween, the blot was developed using Enhanced Chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and visualized by exposure to film.

The results shown in Figure 8 demonstrate that one week old mutant osteoblast cultures secrete significantly more osteoactivin protein than normal osteoblast cultures.

EXAMPLE 12

Detection of Osteoactivin Protein in the Tibia of Normal and *op/op* Mutant Rats

Tibia were harvested from *op/op* mutant rats or from their normal littermates at the indicated weeks of age, immediately frozen in liquid nitrogen, and stored at -80°C until all samples were collected. The frozen bones were then homogenized in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1.0 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate; Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989), 18.38) containing protease inhibitors (Complete tablets, Roche Molecular Biochemicals, Indianapolis, IN), and the insoluble material removed by centrifugation. The concentration of soluble proteins was determined by the method of Bradford (*Analyst. Biochem.* 72:246-54 (1976)) using reagents from Pierce (Rockford, IL). 40 µg of protein from the *op/op* mutant or normal tibia was electrophoresed in a 10% polyacrylamide-SDS gel and then transferred to PVDF membranes by electroblotting. This blot blocked for 1 hr at room temperature in 10% non-fat milk in PBS-Tween and then incubated overnight 4°C in blocking solution containing 0.325 µg/ml chicken anti-rat osteoactivin 551 antibody. Following four washes in PBS-Tween, the blot was incubated at RT for 1 hour in blocking solution containing a 1:5000 dilution of horseradish peroxidase conjugated donkey anti-chicken antibody (Jackson ImmunoResearch, West Grove, PA). Following four washes in PBS-Tween, the blot was developed using ECL reagents (Pierce (Rockford, IL) and visualized by exposure to film. The blot was then stripped of the antibodies and detection reagents and blocked as described. The blot was incubated overnight at 4°C in blocking solution containing 0.75 µg/ml mouse anti-rat tubulin antibody as a control for protein loading between the samples and for the efficiency of blotting across the gel. Following four washes in PBS-Tween, the blot was incubated at RT for 1 hour in blocking solution containing a 1:5000 dilution of horseradish peroxidase conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA), washed, developed, and visualized as described above. The film images from the osteoactivin and β -tubulin experiments were quantitated by scanning densitometry and the data were expressed as a ratio of the osteoactivin to β -tubulin signal in each sample (Figure 9).

These results show that the expression of osteoactivin protein was significantly higher in mutant versus normal rat tibia in all ages examined.

EXAMPLE 13
Inhibition of Osteoblast Differentiation Following Treatment with
Antibodies against Osteoactivin

Osteoblast cultures were established from the calvaria of newborn *op/op* mutant rats or their normal littermates by established methods and plated at a density of 14,200 cells per well of a 24-well plate in MEM α supplemented with 10% fetal bovine serum. Cell culture media was changed every other day and, beginning at day 6 of culture, cells were fed with differentiation medium (MEM α supplemented with 10% FBS, 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate). Beginning at the media change on day 2 of culture and for every subsequent media change, affinity purified antibodies to rat osteoactivin (antibody 551) or control non-immune IgY antibodies were added to the fresh culture medium final concentrations of 4, 20, or 40 μ g/ml. All cultures were terminated at 21 days and analyzed for calcium deposition in the cell/matrix layer using a colorimetric kit from Sigma.

These data (Figure 10) indicate that the antibodies to osteoactivin inhibit rat osteoblast differentiation *in vitro* (as measured by calcium deposition) in a dose dependent manner.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.